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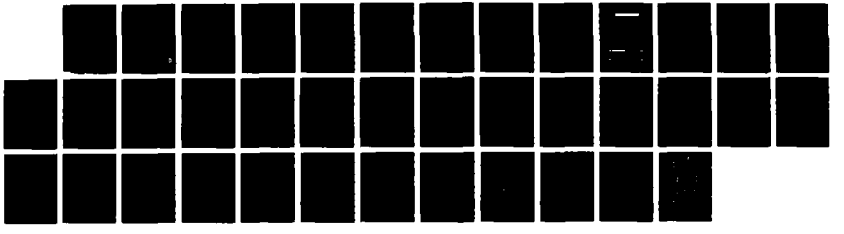

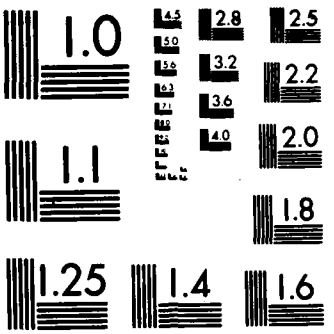

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FINAL TECHNICAL REPORT

CYNTHIA A. LADOUCEUR

ONT/ASEE POSTDOCTORAL FELLOW

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This final technical report consists of two technical papers which were submitted for publication. These two papers represent work performed at the Naval Research Laboratory in Washington, D.C. during the aforementioned time period.

TITLE: Indium-Coated Membranes Versus Nitrocellulose Membranes for use in a Spot Immunoassay

AUTHORS: Cynthia A. Ladouceur and David A. Kidwell

ORAL PRESENTATION: Presented by Cynthia A. Ladouceur on November 17, 1987 at The 1987 U.S. Army Chemical Research, Development, and Engineering Center Scientific Conference on Chemical Defense Research, Aberdeen Proving Ground, MD.

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TITLE: Technologies Used in Mass Screening for Drugs of Abuse

AUTHORS: David A. Kidwell and Cynthia A. Ladouceur

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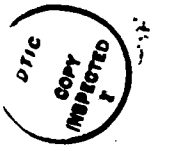
Note: C.A.L. has asked D.A.K. to include a notation in this manuscript to acknowledge the support of ASEE.



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The first paper discusses

INDIUM-COATED MEMBRANES VERSUS NITROCELLULOSE MEMBRANES FOR USE IN A SPOT IMMUNOASSAY.

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ABSTRACT

Immunoassays performed on membranes can be used for the detection of large or small molecular weight substances. The end product of the immunoassay is a spot on the surface of the membrane which is discernable with the naked eye. We tested three different enzyme-substrate systems and colloidal gold with silver amplification. The resulting spots varied in color and in their ability to resist fading. For field testing, the use of multiple and labile reagents is undesirable. When indium-coated membranes are used, antigen-antibody reactions can be visualized with or without development reagents. For the immunoassay on indium-coated membranes, five different amplification schemes were used in order to increase the intensity of the spot. With amplification, the quality of the spots produced on nitrocellulose membranes is far superior to the spots produced on indium-coated membranes. Aluminum, copper, silver, and lead foils were examined in an effort to find substitutes for indium-coated membranes. None of these metal foils were found to be suitable for the visualization of antigen-antibody reactions.

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INTRODUCTION

In 1973, Giaever[1] described an indium slide technique in which antigen-antibody reactions could be observed as a dark area on an indium-coated slide. An indium slide is prepared in a vacuum by evaporating and condensing indium onto a surface. Light scattering by indium spheres produced in the above process can be modified by coating the spheres with thin dielectric layers. These dielectric layers (i.e., proteins) can then be discerned as darker areas on the membrane. Additional dielectric layers applied in tandem increase the light scattering and result in an even darker spot on the membrane.[2,3] For our purposes, the dielectric layers consist primarily of antibodies,

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antigens, and a variety of proteins or protein conjugates which are used to enhance the reaction.

Researchers have used nitrocellulose membranes for the visualization of antigen-antibody reactions since proteins and other substances (i.e., nucleic acids) are strongly and easily adsorbed to the surface of these membranes. Also, these membranes have proven to be useful for several different procedures as described below.

After proteins are electrophoretically separated into their subunit polypeptide chains by gel electrophoresis, the resolved polypeptides can be transferred to nitrocellulose membranes. This transfer from gels to nitrocellulose membranes may be accomplished by the following methods: (1) passive diffusion[4] as described by Southern[5] for the transfer of DNA fragments from agarose gels, or by (2) electrophoretic blotting procedures as described by Towbin et al.[6]. After transfer is accomplished, the bands can be reacted first with specific antibody and then with a second antibody-enzyme conjugate. This, in turn, is reacted with a substrate.[7] In this manner, a great deal of information can be gained regarding the antigenic nature of one or more components of a complex mixture of proteins.

In addition to electrophoretic transfers, proteins are easily adsorbed to nitrocellulose membranes by direct spotting procedures and by dot-blotting.[8,9] Several companies (i.e., Bio-Rad Laboratories, Richmond, CA, and Schleicher & Schuell, Inc., Keene, NH) market an apparatus for dot-blotting. These devices allow up to 96 samples to be applied to a single sheet of nitrocellulose. They also offer a means of concentrating the protein sample on the membrane.

Once proteins are applied to a nitrocellulose membrane, enzyme-linked immunosorbent assays (ELISAs) may be performed on the membrane. Depending on the type of ELISA used,[10] it is possible to test for large or small molecular weight antigens or antibodies.

Unlike indium-coated membranes, nitrocellulose membranes do not allow the researcher to visualize antigen-antibody reactions without the use of multiple and labile reagents. For each membrane, several different amplification schemes were employed to determine the darkest spot that could be produced on the membrane. In this paper, we describe the amplification schemes and the blocking agents which were used; and we present a critical comparison of the two types of membranes for use in a spot immunoassay. We also discuss the possibility of using other metals as substitutes for the indium.

EXPERIMENTAL

The reactions that we employed are illustrated in Figures 1 and 2. The reagents used and the corresponding suppliers are provided in the following listing: (1) nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA and Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); (2) indium-coated membranes (C. L. Burek and J. P. Smith, The Johns Hopkins University, Baltimore, MD); (3) antibodies and enzyme-labeled antibodies (Jackson Immunoresearch Laboratories, Inc., Avondale, PA, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, Pel-Freez Biologicals, Rogers, Arkansas, and Sigma Chemical Company, St. Louis, MO); (4) bovine serum albumin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); (5) human IgG (Sigma Chemical Company, St. Louis, MO); (6) colloidal gold particles (15-20 nm and 40 nm) coated with antibodies (Janssen Life Sciences Products, Piscataway, NJ, and Polysciences, Inc., Warrington,

FIGURE 1

REACTIONS ON INDIUM-COATED MEMBRANES

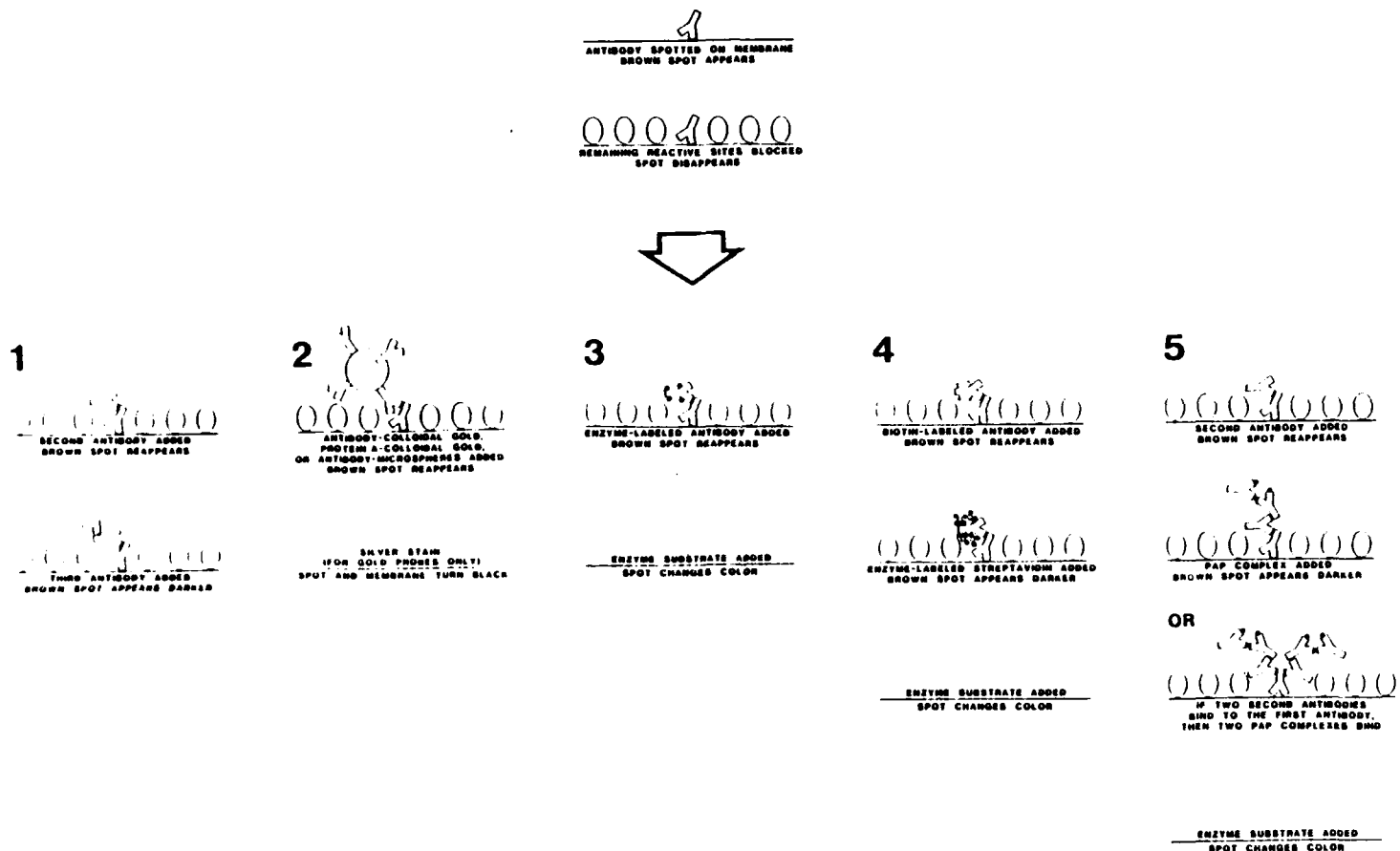
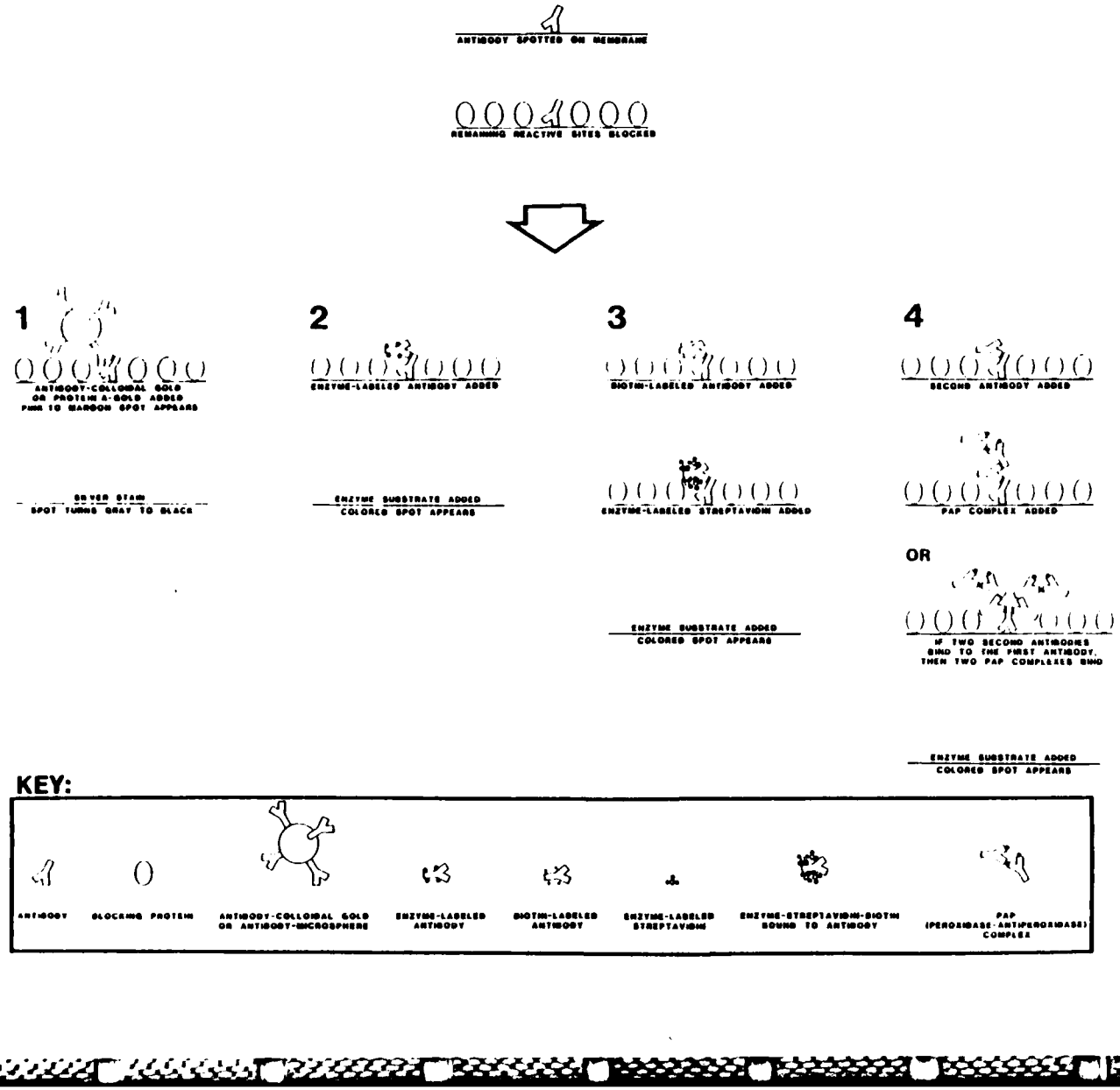


FIGURE 2


REACTIONS ON NITROCELLULOSE MEMBRANES
ANTIBODY SPOTTED ON MEMBRANE
REMAINING REACTIVE SITES BLOCKED
1
ANTIBODY-COLLOIDAL GOLD OR PROTEIN A-GOLD ADDED
PINK TO MAROON SPOT APPEARS
SILVER STAIN
SPOT TURNS GRAY TO BLACK
2
ENZYME-LABELED ANTIBODY ADDED
ENZYME SUBSTRATE ADDED
COLORED SPOT APPEARS
3
BIOTIN-LABELED ANTIBODY ADDED
ENZYME-LABELED STREPTAVIDIN ADDED
ENZYME SUBSTRATE ADDED
COLORED SPOT APPEARS
4
SECOND ANTIBODY ADDED
PAP COMPLEX ADDED
OR
IF TWO SECOND ANTIBODIES BIND TO THE FIRST ANTIBODY, THEN TWO PAP COMPLEXES BIND
ENZYME SUBSTRATE ADDED
COLORED SPOT APPEARS
KEY:
ANTIBODY
BLOCKING PROTEIN
ANTIBODY-COLLOIDAL GOLD OR ANTIBODY-MICROSPHERE
ENZYME-LABELED ANTIBODY
BIOTIN-LABELED ANTIBODY
ENZYME-LABELED STREPTAVIDIN
ENZYME-STREPTAVIDIN-BIOTIN BOUND TO ANTIBODY
PAP (PEROXIDASE-ANTIPEROXIDASE) COMPLEX

PA); (7) colloidal gold particles (5 nm and 20 nm) coated with protein A (Bio-Rad Laboratories, Richmond, CA, Polysciences, Inc., Warrington, PA, and Sigma Chemical Company, St. Louis, MO); (8) three micron diameter acrylic polymer microspheres coated with antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); (9) silver staining reagents (silver lactate, hydroquinone, citrate buffer and fixing solution) (Bio-Rad Laboratories, Richmond, CA); (10) biotin-labeled antibodies and enzyme-labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD); (11) PAP complexes (ICN ImmunoBiologicals, Lisle, IL); (12) enzyme substrates (Bio-Rad Laboratories, Inc., Richmond, CA, and Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). For the horseradish peroxidase-labeled antibodies, the following three substrates were tested: TMB - tetramethylbenzidine, ABTS - 2,2'-Azinobis (3-*ethylbenzothiazoline-6-sulfonic* acid), and 4-CN - 4-chloro-1-naphthol. For the alkaline phosphatase-labeled antibodies, either a mixture of BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate, nitroblue tetrazolium, and tris buffer) or a mixture of naphthol phosphate/fast red was used as the substrate.

Before the addition of each new layer (Figures 1 and 2), the membranes were washed. Indium-coated membranes were washed with running deionized water. After the last water rinse, the membrane was dried with a blow dryer and then examined for spots or dark areas. Nitrocellulose membranes were washed with phosphate buffered saline, pH 8.0 to 8.3 with 0.02% Tween 20 (except for the reactions with gold probes). For the gold probes, the wash buffer consisted of 0.02 M Tris, 0.50 M sodium chloride, pH 7.5 with 0.05% Tween 20. After the last wash, the nitrocellulose membranes were air dried and examined for spots.

In order to block the remaining reactive sites on the indium-coated membranes and avoid nonspecific binding of secondary reagents, we tested the following blocking agents: a 2.5% solution of bovine serum albumin (BSA), and a 2 mg/ml solution of human IgG. For the nitrocellulose membranes, only the BSA solution was used for blocking.

For most of the experiments, two microliters of antibody solution was spotted on the membranes (both types). In one experiment, however, we compared the effect of solution volume and antibody concentration on the spot produced on indium-coated membranes. Two microliters of a solution containing 40 ng of human IgG was spotted on one set of membranes, and six microliters of a solution containing 120 micrograms of human IgG was spotted on another set of membranes.

In an effort to find substitutes for the indium-coated membrane, aluminum, copper, silver, and lead foils were examined. Solutions of horseradish peroxidase-labeled antibodies were spotted in triplicate on both aluminum foil and indium-coated membranes. Each set was incubated with a different enzyme substrate. The substrates tested were TMB, ABTS, and 4-CN.

Eight strips of copper foil, precleaned by swabbing with 1M HCl, were spotted with the following reagents (one set of reagents per strip): (1) antibodies, (2) protein A-gold, (3) BSA, (4) antibodies and protein A-gold, (5) antibodies and BSA, (6) BSA and protein A-gold, (7) antibodies, BSA, and protein A-gold, and (8) protein A-gold with silver staining. The latter reagents were also applied to indium-coated and nitrocellulose membranes.

Five strips of silver foil were first etched with 20% nitric acid. They were then spotted or covered with the following reagents (one set of reagents per strip): (1) antibodies, (2) antibodies and BSA, (3) protein A-gold, (4) antibodies, BSA, and protein

A-gold, and (5) antibodies and protein A-gold. The latter reagents were also applied to lead foil.

RESULTS AND DISCUSSION

Figures 1 and 2 show several reaction sequences that can be used for the detection of antibodies. The procedure can be altered to test for small or large molecular weight antigens. The complexity of each series of reactions and the number of steps involved is easily seen. The term second antibody refers to an antibody that has an affinity for the first antibody adsorbed to the membrane; but the second antibody is derived from a different species than the first antibody. For example, if the first antibody was produced in a goat, the second antibody might be produced in a rabbit. The term third antibody refers to an antibody that has an affinity for the second antibody (i.e., goat antirabbit IgG).

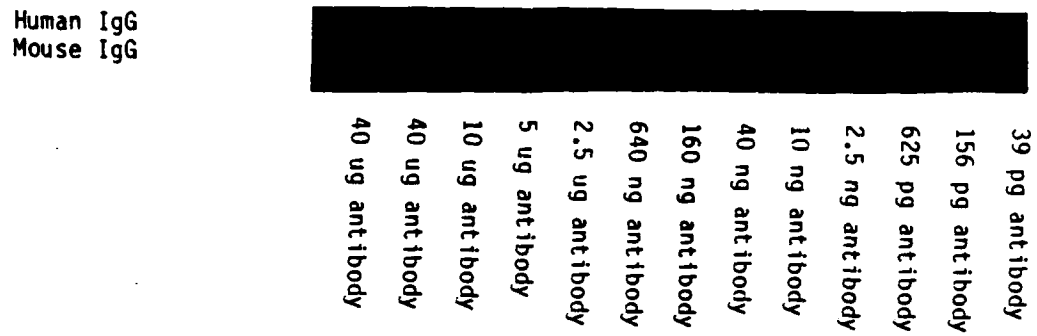
Reactions on Indium-Coated Membranes: For the reactions described in Figure 1, two microliters of an antibody solution are spotted on an indium-coated membrane. The resulting layer of protein on the membrane is visualized as a spot which is darker than the surrounding area. The remainder of the slide is coated with a layer of protein and the spot disappears. The protein used to coat or block the slide is one which does not react with the antigen or antibody being assayed. Blocking reduces nonspecific binding of materials which would reduce the sensitivity of the assay. In order to add additional proteins to the slide, a specific reaction must occur. When the second antibody binds to the first antibody, the bilayer at the reaction site is visualized as a spot which is darker than the surrounding areas. The sensitivity of the test may be improved by the addition of a third antibody as shown in reaction sequence 1 in Figure 1. Reaction sequences 2-5 in Figure 1 show alternative dielectric layers that may be added to the membrane so that the spot appears darker (i.e., antibody-colloidal gold particles, protein A-colloidal gold particles, antibody-microspheres, enzyme-labeled antibodies, biotin-labeled antibodies, PAP complexes, etc.).

Blocking Agents: Previous research studies[2,3] have found that it is better to block the remaining reactive sites on an indium-coated membrane with a protein that has a molecular weight similar to that of the initial protein. Thus, if the first protein is rabbit IgG, it is better to block with another immunoglobulin molecule (i.e., human IgG). We have found this to be the case in our experiments.

Single Dielectric Layer Applied to Membrane: Antibodies were used to test the effect of applying a single dielectric layer to an indium-coated membrane (see Figure 3). After spotting indium-coated membranes with successive dilutions of human IgG and mouse IgG, the lowest concentration of either of these antibodies that could be seen as a discrete spot was 40 ng. The spots produced in this manner are brown against a tan background. They are somewhat difficult to discern even when viewed with the aid of a light box. An additional problem is that the water wash and uneven drying result in smudges on the membrane. The smudges can obscure the spots if the two are in close proximity. This is a disadvantage of using indium-coated membranes, but it is not a problem on nitrocellulose membranes.

Effects of Solution Volume and Antibody Concentration: Solution volume and antibody concentration has an effect on how easily one discerns a spot on an indium-coated membrane. Smaller volumes and concentrations appear to produce a more discrete spot than larger volumes and higher concentrations. At higher volumes and concentrations, the spots on the membrane tend to be more diffuse and smudging occurs.

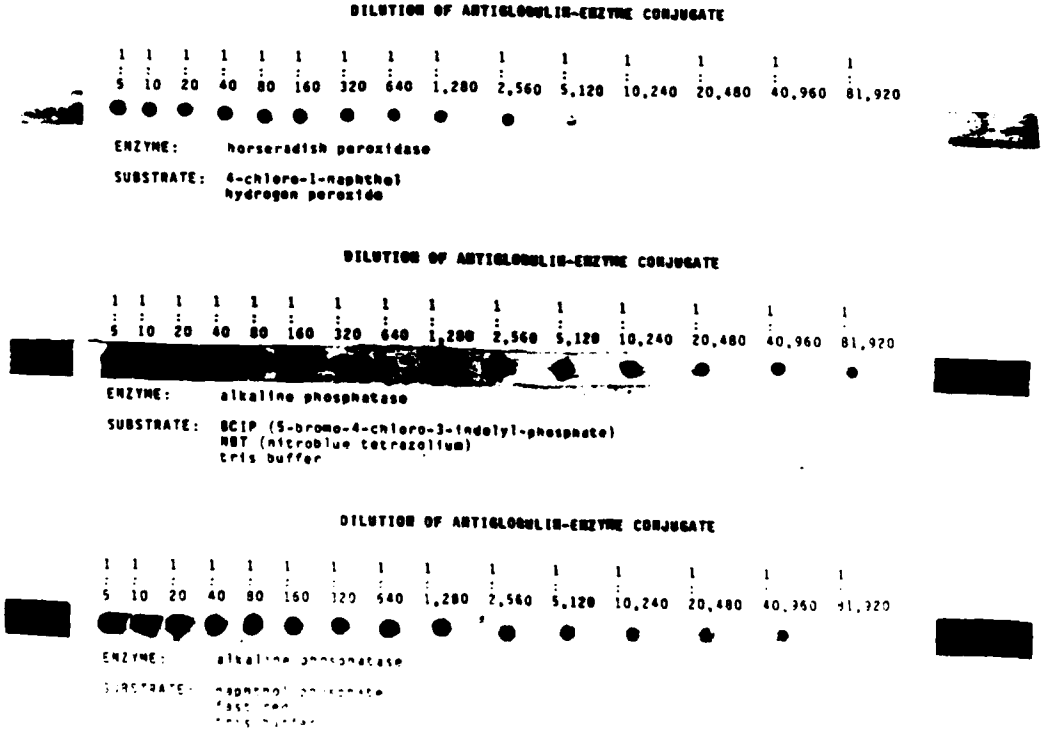
FIGURE 3
ANTIBODY SPOTTED DIRECTLY ON AN INDIUM COATED MEMBRANE



Note: Each quantity represents the maximum amount of antibody that is available for binding to the membrane per spot.

FIGURE 4
ANTIBODY-ENZYME CONJUGATE SPOTTED DIRECTLY ON A NITROCELLULOSE MEMBRANE AND DETECTED WITH SEVERAL DIFFERENT ENZYME SUBSTRATES

Purple spots were produced on the first two membranes and red spots were produced on the third membrane.



Effects of Colloidal Gold Particle Size: When neat solutions of colloidal gold particles coated with antibodies were spotted directly on indium-coated membranes, the 40 nm size colloidal gold particles (Janssen Life Sciences Products, Piscataway, NJ) appeared to produce the darkest spot as compared to the smaller colloidal gold particles tested. These spots, however, were not as discrete as the same sample applied to nitrocellulose membranes.

Effects of Silver Staining: When gold probes were used on indium-coated membranes and then exposed to silver staining reagents, both the spot and the membrane tended to turn black or gray-black. Thus, this is not a useful method for amplifying the reaction.

Multiple Dielectric Layers Applied in Tandem: Other researchers[2,3] have found that the addition of several layers of protein applied in tandem to indium-coated membranes increases the light scattering which, in turn, makes the spot appear darker. We did not find this to be the case.

Monitoring Binding: In order to monitor whether or not antibodies were actually bound to the indium-coated membrane, enzyme-labeled antibodies were applied to the membrane. This was followed by submerging the membrane in a substrate that was appropriate for the enzyme. Degradation of the substrate at the site of antibody-enzyme attachment to the membrane results in a colored spot. This experiment was repeated several times using horseradish peroxidase-labeled antibodies plus three different enzyme substrates (TMB, ABTS, and 4-CN). The use of enzyme-labeled antibodies and enzyme substrates was found to be an effective way of monitoring whether or not a first, second, or third antibody was actually bound to the indium-coated membrane. Of the enzyme-substrate systems tested, alkaline phosphatase with BCIP/NBT appears to produce the most clearly visible spot on the membrane. The spot is a purple-blue color, but one must orient the membrane toward the light in order to see the spot.

Other Metals: Aluminum, copper, silver, and lead were tested as alternates for the indium. None of these metals appear to be useful for membranes upon which antigen-antibody reactions can be performed.

Reactions on Nitrocellulose Membranes: The reactions shown in Figure 2 for nitrocellulose membranes are very similar to those in Figure 1. For each reaction sequence, it is possible to have two second antibodies bind to the first antibody. However, this is only illustrated for the PAP complexes. The spots produced on nitrocellulose membranes are discrete and easily seen against the white background of the membrane. The colors of the spots vary with the reagents used (see Figure 4). Since the original protein concentration was unavailable, the spots shown in this figure are labeled as dilutions of the neat material. It is possible to detect this antiglobulin-enzyme conjugate even after it has been diluted 1:81,920.

For the reactions on nitrocellulose membranes, we have employed both horseradish peroxidase and alkaline phosphatase-labeled antibodies and several substrates for these enzymes. TMB, ABTS, and 4-chloro-1-naphthol were the substrates used for the horseradish peroxidase-labeled antibodies. BCIP/NBT and naphthol phosphate/fast red were the substrates used for the alkaline phosphatase-labeled antibodies. With any of these reagents, we can produce a discrete and clearly visible spot on nitrocellulose membranes.

The intensity of the spot depends on the concentration of the material being

detected. For example, with high concentrations, one sees a red spot on the nitrocellulose membrane when naphthol phosphate/fast red is used as the substrate. At low concentrations, one sees a pink spot on the nitrocellulose membrane. For the nitrocellulose membranes, we have also used protein A-gold and antibody-gold particles with and without silver staining amplification. With the protein A-gold and antibody-gold reagents and silver staining amplification, high concentrations of antibody or antigen on the membrane result in a spot that is a deep black color. This spot resembles one that would be produced with a black felt-tip marker. For very low concentrations, this spot is dark gray to light gray in color. Improper handling of silver staining reagents affects the assay results. For example, if the silver lactate reagent is exposed to light for an extended period of time, the spots on the membrane will be gray instead of black (even though the antigen or antibody on the membrane is in highly concentrated form).

Comparison of Indium-Coated and Nitrocellulose Membranes: There are two major differences in the reaction sequences shown in Figures 1 and 2. With the reactions in indium-coated membranes, theory predicts that spots should appear at every state of the reaction sequence except for the blocking step. Furthermore, as additional dielectric layers are added to the membrane, the spot should appear progressively darker. For the nitrocellulose membranes, the earliest stage that a spot can be seen is shown in Figure 2, reaction 1 (after the addition of antibody-colloidal gold or protein A-colloidal gold). For all the other reaction sequences performed on nitrocellulose membranes, the spot is not visualized until the very last step of the procedure (after the enzyme substrate is added). If the reactions on indium-coated membranes did follow the predicted course shown in Figure 1 and spots could be seen at all steps (excluding the blocking step), than this type of membrane would have the distinct advantage of being able to monitor each step of the reaction sequence. With the enzyme-linked reactions on nitrocellulose membranes (Figure 2, reactions 2-4), one has to wait several hours until the substrate is added before a spot appears on the membrane. Unfortunately, we have found that the reactions on indium-coated membranes do not follow the predicted course of darkening as each new dielectric layer is added to the membrane. Even if this darkening does occur, the change is not great enough for one to discern with the unaided eye.

The second major difference that we observed is in the quality of spots on the membranes. The spots on the nitrocellulose membranes are clear, discrete, do not fade readily, and can be a variety of colors depending on the reagents used (i.e., blue-green, purple, red, or black). The very darkest spots obtained on the indium-coated membranes are a light to dark brown color and are seen against a tan surface. A shiny purple-blue spot may be seen on the surface of these membranes when certain enzyme-substrate systems are used; however, one has to orient the membrane towards the light in such a manner that the spot is clearly visible. We have found that the darkest spots produced from reactions on indium-coated membranes are much more difficult to discern with the unaided eye than the darkest (black) spots produced from reactions on the nitrocellulose membranes. Furthermore, the indium-coated membranes scratch easily and spots can be wiped off the membrane by gentle blotting with tissue.

CONCLUSIONS

When performing immunoassays on membranes, the spots should be clear, discrete, and permanent. This occurs on nitrocellulose membranes but not on indium-coated membranes. In addition to the poor quality spot produced on the surface of indium-

coated membranes, these membranes are easily scratched and the reaction results could be wiped off by blotting the membranes with tissue. Nitrocellulose membranes are readily available from several suppliers. The indium-coated membranes are not readily available. In addition, nitrocellulose-based immunoassays are a more mature technology and have proven to be useful for a variety of applications as described earlier in this report. Based on our experimental findings, we recommend the use of nitrocellulose membranes for use in spot immunoassays.

ACKNOWLEDGMENT

C. A. Ladouceur would like to thank the Office of Naval Technology/American Society for Engineering Education Postdoctoral Fellowship Program for their support.

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Cont'd
The second paper pertains to

TECHNOLOGIES USED IN MASS SCREENING FOR DRUGS OF ABUSE.

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II-15

INTRODUCTION

In an effort to combat drug abuse and provide for a safe work place, widespread testing of urine for drugs of abuse has become a reality. Drug screening laboratories have emerged in a wide variety of environments. These include government and industrial facilities, emergency rooms, psychiatric centers, and substance abuse centers.

The Department of Defense conducted over 27 million urine tests in 1986. Disciplinary action is often taken on the basis of a urine test alone. To stay within budgetary constrains, the tests must be inexpensive. Yet society, through the courts, has demanded that urine tests meet accepted scientific criteria for validity.

A good urine screening program must employ two independent tests, and these must be in agreement before a sample is considered positive. The first test is a screening test. This test serves to filter out the large number of negative samples and to indicate what drug should be analyzed in the confirmation test. To meet cost criteria, the first test is inevitably based on thin layer chromatography or an immunoassay. Since the second test is performed on less than 5% of the samples, it can be a more precise and expensive test. Gas chromatography/mass spectrometry (GC/MS) is generally used as the confirmation test.

This paper reviews some of the techniques used in mass screening for drugs of abuse and discusses the principles, advantages, and disadvantages of each.

CUT-OFF LEVELS

In order to state that a sample is positive for drugs, at least three criteria must be met: (1) the sample must be positive by the screening test above a set cut-off level, (2) the sample must be positive by a confirmation test above a set cut-off level, and (3) the quantitation by the two tests must agree.

Why are cut-off levels important? Does any amount of drug in a person's urine label him/her as a drug user? Cut-off levels are set to ensure that the screening and confirmation tests are sensitive enough to reliably detect the presence of a drug. Setting the cut-off level too low may give so many positives in the screening test as to overwhelm the slower and more costly confirmation by GC/MS. However, often cut-off levels are set much higher than is necessary based on the sensitivities and specificities obtained by the two tests. The higher cut-off levels are set with consideration of the workload of the laboratory and the possibility of passive ingestion of drugs. For example, Table 1 lists three common products and the drugs of abuse they contain.

Table 1 - Common Products That Contain Controlled Substances

Product	Drug Contained
Poppy Seeds	Morphine (0.2% w/w)[1]
Cow's Milk	Morphine (1 ng/L)[2]
Vicks Inhaler[36]	l-methamphetamine (d-methamphetamine is the abused drug)

If cut-off levels were set slightly above the detection limits, and with the low picogram (10^{-12} g) sensitivities available by modern instrumentation, most people would be classified as drug users. The substances in Table 1 are illegal substances in the appropriate context.

The cut-off levels used by the Navy to avoid unjust accusations of people and the substances screened are given in Table 2[3]. These levels are similar to those that will be implemented in government civilian testing[4].

A person could not obtain a urine positive from milk and milk products since the amount of morphine is extremely low. Poppy seeds, on the other hand, can produce urine positives. Poppy seeds have widely varying morphine content depending on the strain of plant and the geographic and climatic conditions for plant growth. Early reports indicated that poppy seeds would not cause a urine positive[5-7]. More recent data indicates that poppy seeds can give morphine levels of as high as 3000 ng/ml[8,9].

These more recent reports have caused a reevaluation of the cut-off levels used by the military. Current Navy policy is to report any level above the 300 ng/ml cut-off as an opiate positive. However, all reports of the presence of opiates include the message to contact the laboratory submitting the report so that the drug levels may be examined and interpreted. Other factors, such as drugs in the individual's possession, must be considered to indicate drug abuse if the morphine level is below 6000 ng/ml. Consideration of other factors are also mandated by the Health and Human Services guidelines for civilian testing.

The Navy is currently evaluating its testing of opiates and will soon modify the guidelines so that morphine levels below 6000 ng/ml will be tested further. The presence of 6-acetyl morphine, a heron metabolite not found in poppy seeds, will be tested by GC/MS. If present the use of heroin would be confirmed. This metabolite is not present in the urine of all heroin users in detectable quantities. Some individuals will escape detection by this procedure but no innocent people would be identified incorrectly. The chance of false identification far outweighs the few individuals that would not be detected.

Table 2 - Current Cut-off levels used by the Navy

Abused Substance	Substance Detected			
	RIA	Cut-off Level	GC/MS	Cut-off Level
Marijuana	THC cannabinoid metabolites	100 ng/ml	delta-9-THC carboxylic acid	15 ng/ml
Cocaine	Benzoylecgonine Cocaine, and Ecgonine	300 ng/ml	Benzoylecgonine	150 ng/ml
Amphetamine	Amphetamine	1000 ng/ml	Amphetamine Methamphetamine	500 ng/ml
Barbiturates	Barbiturates	200 ng/ml	Phenobarbital Butabarbital Amobarbital Secobarbital Pentobarbital	200 ng/ml
Opiates	Morphine	300 ng/ml	Morphine Codeine	300 ng/ml
Phencyclidine	PCP and metabolites	25 ng/ml	PCP	25 ng/ml

The optical isomer of methamphetamine that is abused as 'speed' is d-meth mphetamine. Vicks Inhaler contains 50 mg of l-methamphetamine which has little central nervous system stimulating activity. Current urine tests do not distinguish between these isomers because both are illegal to possess and use. Vicks Inhaler is excepted under section 1308.22 of the Federal Code of Regulations.

A typical dose of d-methamphetamine is 15 mg so that Vicks Inhaler contains 2-3 times the street dose of methamphetamine. About 85% of the dose of methamphetamine is metabolized to amphetamine before excretion in the urine[10]. The cut-off levels for amphetamine were set at 500 ng/ml as a precaution so that a person using a cold remedy would not be called positive for drug abuse. Tests were performed on use of Vicks Inhaler at more than the recommend levels and urine values for methamphetamine and amphetamine did not approach the 500 ng/ml cut-off level[11]. More recent indications are that levels of up to 800 ng/ml can be reached by use of Vicks Inhaler. Therefore, the cut-off levels are being reevaluated.

One could abuse a Vicks Inhaler by eating it or extracting the methamphetamine and ingesting the extract. Likewise, morphine could be extracted from poppy seeds and injected or converted to heroin and injected. In both cases, a urine positive may be produced that would have scientific validity since the methamphetamine or morphine would be present in the urine sample. However, there is no distinction between abuse of a substance legally obtained and abuse of the same substance illegally obtained.

QUANTITATION

Many drugs are metabolized before being excreted in the urine. For example, cocaine is metabolized almost 100% to benzoylecgonine and ecgonine before being excreted[10]. Methamphetamine is metabolized 85% to amphetamine and heroin (diacetylmorphine) is metabolized to morphine[10]. Most immunologically based screening tests cross-react to some extent with structurally similar compounds, often the metabolites of the drugs. Therefore, the cut-off levels are set differently for the screening tests versus the confirmation test by GC/MS, which is structurally specific.

Quantitation of the presence of the drugs by both the screening test and the confirmation test lends more weight to the validity of the positive. Nevertheless, there may not be an exact agreement between the two tests. Normally GC/MS indicates less drug than immunoassay due to metabolites being present. For example, a screening test may indicate 400 ng/ml of morphine and the GC/MS data may indicate 300 ng/mL However, if the screening test indicated a 300 ng/ml of morphine but the GC/MS confirmation test indicated 400 ng/ml of morphine there would not be an agreement between the two quantitations and a possible mix-up of the sample may have occurred.

It is more common for an immunoassay to indicate a high drug level and the GC/MS confirmation to find no drug. In these cases, cross-reactivity with another compound is likely to have occurred. That sample would be called negative since no drug was found in the confirmation test; therefore, the quantitation was not above the cut-off level. No adverse publicity would ensue on the individual donating that specimen. The importance of the confirmation test can not be over stressed because of the occurrence of cross-reactivity in many screening tests.

SCREENING METHODOLOGIES

The screening test does not need to be infallible[12]. It may call some samples positive for a drug when none was present (i.e. a false positive); but it should detect all those samples that have drugs present (i.e. no false negatives). Therefore only two requirements must be met by the screening test: (1) it must be specific enough to avoid an excessive number of false positives, and (2) it must have a sensitivity equal to or less than the cut-off levels given in table 2.

Any of the following specimens are used for the detection of drugs of abuse: (1) urine, (2) serum, (3) saliva, and (4) hair. Drug levels may vary depending on the body fluid used for the test. Urine is typically used for most mass drug screening programs because it is the least intrusive sampling method, and can detect drugs for extended periods after drug use.

The military uses urine for their drug tests. There are strict requirements on the collection of samples. Direct observation of the donation of the sample is required to prevent possible substitution or adulteration of the sample. However, Executive Order #12564 signed by President Reagan on September 15, 1986 directing civilian testing requires privacy during sample donation. This allows the possibility of

substitution or adulteration of the specimen to mask drug use. To prevent substitution of urine samples, the following procedures are required (1) dying of the water at the collection station, (2) careful listening for the sample being donated, (3) monitoring the temperature of the donated specimen, (4) removal of unnecessary garments, and (5) positive identification of the individual[4].

The following sections discuss the two types of screening tests which are commonly employed: (1) thin layer chromatography, and (2) immunoassay (radioimmunoassay, enzyme multiplied immunoassay, and fluorescent polarization immunoassay). For each assay, we will examine the principles, advantages, and disadvantages.

Thin Layer Chromatography (TLC)

TLC was first described in 1938[13]. In TLC an adsorbent (stationary phase), such as silica gel, alumina, or an ion exchange resin, is uniformly coated on a glass plate or plastic film. Mixtures of known drug standards or extracts of urine are applied as spots to the plate. The spotted end is placed in a closed container with enough solvent (mobile phase) to wet the bottom of the plate. The solvent is drawn up the plate by capillary action carrying with it the spotted materials. The materials interact with the stationary phase to different degrees and are separated while migrating up the plate. The final position of the spot on the plate is indicative of the drug. The plate may be sprayed or dipped in a number of reagents to aid in identifying the species present.

TLC requires extraction of the urine sample before the analysis. This is more labor intensive than immunoassay which can be run on the sample directly. Nevertheless, TLC can be as cost effective as immunoassay since TLC can detect many drugs simultaneously whereas immunoassay can detect only one drug or drug class per test[14].

The detection levels for TLC are in the 0.5-1.0 μg/ml range for most substances[15]. These sensitivities are much higher than the cut-off levels listed in Table 2 and are therefore not adequate for military testing. TLC is often employed for testing drugs where immunoassay are unavailable. Because of the low sensitivity of TLC, not every drug user is detected. However, not every drug user must be punished to deter drug use. One of the most widely used commercial systems is marketed by Analytical Systems under the name Toxi-Lab[36]. This system is supplied with an extensive compendium of drugs and their TLC behavior. Toxi-Lab often is employed in hospitals as a rapid screening method in cases of suspected drug overdose.

Toxi-lab consists of two tests. One test for basic drugs such as opiates and amphetamines, and one test for acidic drugs such as barbiturates. The procedure is as follows: urine is first measured into a vial containing an extraction solvent, a colored dye and buffer salts. After mixing, the solvent layer is removed and placed into disposable cups along with a absorbent disk. The solvent is evaporated and the extracted compounds concentrated onto the disk. The disk is removed from the cup and placed into a precut hole in a silica-impregnated, fibrous, TLC plate. The TLC plate can hold two disks and also contains four other columns containing drug standards. Blank plates, without standards, are also available. The TLC plate is developed in a solvent system and the solvent evaporated. For the acidic drugs, the plate is then exposed to formaldehyde fumes, and the colors developed in sulfuric

acid. For further confirmation the sulfuric acid is removed with a water wash and the fluorescent examined under long-wavelength ultraviolet light. Then the compounds are stained with a modified Dragendorff's reagent. The positions and characteristic colors of the spots at each stage of the staining procedure are indicative of the drugs present. All must be correct before a sample is presumed positive for that drug. Toxi-Lab can detect over 200 drugs. Several common drugs of abuse such as marijuana, LSD, and cocaine are not detected by the standard Toxi-Lab procedure.

For the acidic drugs, Toxi-Lab detects primarily the barbiturates and barbiturate like compounds. The sample is treated the same as for the basic drugs as described above until the formaldehyde step. Instead of formaldehyde, the plate is stained with diphenylcarbazone, silver nitrate and then with mercuric sulfate. The fluorescence is then examined under long wavelength ultraviolet light. Again, the colors and positions of the spots in each step are characteristic of the drugs present.

We have evaluated Toxi-Lab for the analysis of over 400 randomly-selected Navy urine samples. These samples were being tested to determine if other drugs may be present that were not detected by immunoassay. These samples were drug-free for the drugs at the cut-off levels given in Table 2. Most Navy samples are not tested shortly after collection. They are often mailed by surface mail from distant places and may be weeks old before testing. This has not been shown to be a problem for immunoassay. However, for TLC false positives frequently occur. Degraded urine, presumably from bacterial contamination since no preservatives are added, caused over a 30% false positive rate for the barbiturates. Often a spot would appear that would be identical in all respects to phenobarbital but no phenobarbital would be detected by GC/MS. Also, due to the simple extractions employed in Toxi-Lab, many neutral compounds would be co-extracted with the basic drugs. These led to large brown streaks that obscured any compound present and reduced the sensitivity ten fold from that achievable with fresh samples.

Immunoassays

In order to understand the principles involved in immunoassay, one must be familiar with several terms used in immunology. An antigen is any molecule which is able to elicit an immune response. The term immunogenicity refers to the extent to which an antigen elicits an immune response. Antigens vary widely in their chemical composition. Typically, large proteins and polysaccharides tend to be highly immunogenic; nucleic acids, lipids, steroids, and drugs tend to be weakly immunogenic or nonimmunogenic unless they are coupled to an immunogenic carrier such as a large protein. An antibody is a protein molecule which is produced in response to an antigen and is able to recognize and bind to the specific antigen that stimulated its production. Proteins which have antibody activity are known as immunoglobulins which are classified by their heavy chain structure. Since immunoglobulin G (IgG) is the most abundant in serum, it is the primary antibody used for the immunoassay discussed in this text. The IgG molecule will be depicted as a Y shape. The two antigen binding sites of the molecule are present at the ends of the Y. For a more detailed description antibodies refer to the text by Steward[17].

The sensitivity of a drug immunoassay is the lowest concentration of the drug that can be detected reliably. The specificity of the drug immunoassay refers to the

ability of the antibodies to distinguish one drug from another. Cross-reactivity refers to the binding of an antibody to an antigen other than the one that elicited its production (i.e., binding to molecules which are similar in structure). For the detection of drugs cross-reactivity can be both a help and hindrance. It is helpful because often a class of compounds can be detected, as in the case of barbiturates. This allows the testing of many related compounds without the need to preform multiple tests. Cross-reactivity can be a hindrance in correlating the quantitation given by the immunoassay to the more specific GC/MS especially when metabolites are present.

Three different immunoassay are described in the following sections.

Radioimmunoassay (RIA)

Radioimmunoassay was first described in 1959 by Rosalyn Yalow as a method to detect insulin[18]. For her work in this area, she received the 1977 Nobel prize in Medicine or Physiology. To perform the test, a known amount of radioactively-labeled drug (antigen) is mixed with a small quantity of urine (a few hundred microliters). Then antibody to the drug is added. For a successful test, the antibody should not be able to distinguish between that radioactively-labeled drug and that drug which may be present in the urine. The antibody and drug-antibody complex are separated from the urine and the radioactivity measured. The more radioactivity bound to the antibody the less drug present in the test sample. The principle of RIA can be seen schematically in Figure 1.

The separation of bound drug from unbound drug may be performed in many ways[19]. Three methods are frequently used. One method relies upon the addition of a second antibody which is directed against the first antibody. Because the second antibody can bind two first antibodies, a large insoluble molecule is formed. This precipitate is pelleted by centrifugation. The supernatant containing the unbound antigen is discarded. Then the radioactivity in the pellet is counted directly in the tube.

Another method for separating the bound from unbound antigen relies upon binding the antibody to the walls of the reaction vessel. After the initial drug-antibody reaction, the unbound materials are poured out. The radioactivity bound to the antibodies which are coated on the walls of the tube is then determined.

The last method relies upon adsorption of the unbound antigen onto activated charcoal. The charcoal is coated with a dextran polymer which forms a matrix and allows only the smaller antigens to pass through and be absorbed onto the charcoal. The charcoal is then removed, and the radioactivity in the urine or the charcoal is determined.

A typical plot of radioactivity observed versus concentration of antigen is shown in Figure 2. This curve is identical to an acid-base titration curve. The principles behind both are similar. The linear working-range is on the S-shaped portion of the plot. This curve may be made linear as shown in the bottom of Figure 2[20].

The advantage of RIA lies in its sensitivity; 10^{-12} - 10^{-15} M of antigen can be routinely determined. This sensitivity is the result of the low radioactive background

of most materials and the high sensitivity of radioactive measurements.

For most RIA work a short-lived isotope, i.e., I^{125} (half-life = 60 days) or tritium (half-life = 12.5 years) is used. Even with these short-lived isotopes, 10^7 atoms of I^{125} stand mute for every 100 disintegrations that occur per minute. Radioisotopes with shorter half-lives may be used to increase sensitivity, but the shelf-life of the radiolabeled antigen is correspondingly reduced.

Figure 1 depicts the principle behind RIA. Considering the principle of competitive binding, there are few, if any, adulterants that would allow the antibody to preferentially bind to the radiolabeled-drug and not to an drug in the sample. Adulterating ones urine by adding antidrug antibodies or radioactively-labeled drugs would cause false negatives, but these materials would not be readily available to the average drug abuser. The addition of common adulterants such as salt, organic solvents, acids or bases to prevent antibody binding would prevent the antibody from binding to both radiolabeled-drug and the drug present in the urine. For example, making the urine quite acidic (pH <2) causes a false positive for all drugs tested which certainly raises suspicion about adulteration. This is a result most drug abusers would wish to avoid.

The disadvantage of RIA is the risk of exposure to radiation and the restrictive laws regulating the distribution, use, and disposal of radioisotopes.

Enzyme Multiplied Immunoassay Technique (EMIT)

In 1972, Rubenstein developed the homogeneous assay upon which EMIT[36] is based[21]. A common enzyme system used for EMIT is shown in Figure 3. The enzyme, glucose-6-phosphate dehydrogenase (G6PD), uses nicotine adenine dinucleotide phosphate (NADP) as a cofactor to oxidize glucose and reduce the NADP. The reduced NADP absorbs UV light at a longer wavelength than the oxidized form. The activity of the enzyme can be measured as a rate of increase in absorbance due to the production reduced NADP.

The principle behind EMIT can be seen in Figure 4. The binding of the antibody to a enzyme-labeled drug decreases the activity of that enzyme. Only a few enzymes with the active site close to the surface show this effect[22]. To perform the assay, the rate of turnover of the enzyme must be measured. The decrease in activity is measured as a decrease in absorbance after a set period of time compared to a standard. If no drugs are present in the test sample, then all the enzyme-labeled drug is bound and the activity of the enzyme is reduced. The bound enzyme-labeled drug produces little reduced NADP and the absorbance at 340 nm is correspondingly low. If a quantity of drug is present in the test sample, then the enzyme-labeled drug with the drug-enzyme conjugate for the antibody binding sites (recall the discussion of RIA above). This releases some of the enzyme-labeled drug, restores its activity, and produces more reduced NADP. Therefore the absorbance at 340 nm increases.

The sensitivity of EMIT could be higher than RIA since all the signal is produced at a given time. In practice, the binding of the antibody to the enzyme-labeled drug does not eliminate the activity. Therefore, the background signal is higher and the sensitivity is reduced from that theoretically possible.

The advantage of EMIT over RIA is that no radioactivity is involved. This makes disposal of waste products relatively easy. Also, the shelf-life of the reagents is increased since no radioactive decay is present and the signal can be started and stopped. Labor can be saved since the assay is preformed without a separation step.

A disadvantage of EMIT is that it cannot be used if the test sample is cloudy or has interfering substances that absorb at 340 nm. Also, EMIT employs enzymes which are quite sensitive to interferences.

EMIT is the one test that has had the most publicity about its susceptibility to adulteration. Like RIA, an adulterant that prevented binding of the antibody to the drug-labeled enzyme would generate a false positive. Unlike RIA, EMIT is vulnerable by reducing the activity of the enzyme or changing the NADP cofactor. Some of the common adulterants that may be used to generate a false negative are listed in Table 3[23]. Since the urine is diluted 20 fold with buffer, a very high salt concentration, acid concentration or base concentration are needed to achieve a false negative. Even with a saturated salt solution, a sample with a high concentration of drug will still product a positive by EMIT. Adulteration with salt, acids, or bases would be readily detected by simple tests (specific gravity or pH measurements) done on the urine after collection.

Table 3 - Common Adulterants used to Generate False Negatives in EMIT

Salt
Acids such as vinegar
Bases such as lye
Oxidants (bleach)
Enzyme inhibitors (heavy metals)

EMIT is quite sensitive to oxidants such as bleach. The presence of oxidants are much harder to test than pH or specific gravity. Oxidants oxidize the NADPH formed by the enzyme back to the NADP. Since the formation of NADPH from NADP is measured, its conversion back to NADP by bleach generates a false negative. Experiments were done to determine if this is a likely mechanism by destroying the oxidant with ascorbic acid before testing a spiked sample. After adding the ascorbic acid, the sample again tested positive.

EMIT is also quite sensitive to adulterants such as enzyme inhibitors. Specific competitive inhibitors of glucose-6-phosphate dehydrogenase exist that generate false negatives if present in the urine sample. These adulterants would also be quite difficult to detect.

One system that could be used to test for adulteration in urine tests that employ EMIT technology is another enzyme test. The urine would be spiked with a compound which is not normally present in the urine, and this compound could be quantitation done by EMIT. If the results do not agree with the amount spiked, adulteration would be suspected and the sample could be tested by an alternative procedure. However, this increases the cost of the test by requiring one more assay.

Fluorescent Polarization Immunoassay (FPI)

The principles of fluorescent polarization were first developed by Perrin in the 1920s[24]. Its application to the detection of antigens bound to antibodies was first described by Dandliker and Feigen in 1961[25]. The principles and practice of fluorescent polarization and its application to biological systems have been the subject of several review articles[26-28].

The basis of FPI can be seen in Figure 5. If a polarized light beam excites a stationary, fluorescent molecule, the molecule also will emit the light polarized at a longer wavelength. Generally, smaller molecules such as drugs rotate faster than larger molecules such as antibodies. An antibody, binding to the smaller, fluorescent molecule would make a large complex with a slower rotational period. This large complex would not rotate significantly before fluorescence of the molecule had occurred; therefore, the polarization of the initial exciting light would be retained.

The sensitivity of FPI is somewhat less than can be achieved by RIA and EMIT, but it is sufficient for most drug assays. The sensitivity is limited by the theoretical maximum of polarization being 0.4 (due to the random distribution of molecules). Sensitivity is also limited by the inherent fluorescence of the sample. This is especially severe if proteins, as in blood plasma, or certain vitamins are present.

The shelf-life of the reagents in FPI is increased over both EMIT and RIA as no radioactivity or enzymes are involved with this analysis. Like EMIT, labor can be saved since the assay is performed without a separation step.

Fluorescent polarization is sensitive to a number of adulterants[28]. Considering the principle behind fluorescent polarization, any high molecular weight material that nonspecifically binds the fluorescent label would generate a false positive. This nonspecific binding would reduce the rotation of the molecule and increase the polarization just as if the antibody had bound to the drug-labeled fluorophore. Proteins are known to interfere with fluorescent polarization in this manner.

If the fluorescent lifetime were significantly reduced, even unbound molecules would appear to be stationary. Heavy metals are efficient fluorescent quenchers that can reduce lifetimes in high enough local concentrations. These should generate false negatives with this assay.

Unlike RIA, one label (the fluorescent dye) is used to generate a signal. Antibodies could be raised to this molecule. If these antibodies are placed in the urine they would generate false negatives for all drugs. In the case of RIA, an antibody for each drug of abuse would be needed. These antibodies are not readily available.

Fluorescent molecules present in the urine, such as some vitamins, can interfere with this assay. These materials emit enough light to mask the relatively weak polarized light emitted by the labeled drug.

The TD_X system used by Abbott Laboratories reduces or eliminates all these known interferences by a large 250-fold dilution of the urine in buffer before an assay is performed[35]. We have tested samples with large amounts of protein, salt, and iodide (as a fluorescent quencher) without generating a false negative. Fluorescent materials in the sample still pose a problem. Abnormally high levels of fluorescence

are indicated to the operator by the TD_X instrument. The sample can then be tested by alternative techniques.

CONFIRMATION TESTS

Hoyt surveyed legal professionals, forensic experts, and arbitrators about the legal defensibility of various test methods[29]. GC/MS had almost a perfect score for complete defensibility. Other confirmation tests, those using different technology than the screening test, were less acceptable but most felt they could be defended in court. The military requires all confirmations to be performed using GC/MS.

Gas Chromatography/Mass Spectrometry (GC/MS)

Gas chromatography is very similar in operating principle to TLC. GC consists of two parts: (1) a glass capillary column which is coated with a stationary phase, and (2) an inert gas as the mobile phase. The sample is vaporized in a heated inlet and is transported through the capillary column by the inert gas. Like TLC, the components are separated on the basis of their affinities for the stationary phase. The components with the lowest affinities elute first.

Various steps listed in Table 4 must be done before a sample can be analyzed by GC or GC/MS. Since these steps are labor intensive, the instrumentation is expensive, and the analysis is time consuming, GC/MS is only cost effective for confirmation.

Table 4 - Steps in a GC/MS Analysis

1. Measure sample
2. Add internal standard
3. Adjust pH
4. Extract sample
5. Concentrate extract
6. Derivatize extract (optional)
7. Run GC/MS
8. Analyze data

The use of mass spectrometry with gas chromatography had its beginnings in the early 1960's when Ryhage[30] and Watson and Biemann[31] successfully connected a GC to a mass spectrometer. Prior to that time, fractions were purified by GC, trapped, and introduced individually into the mass spectrometer. Major advances in instrument automation and computer capabilities have occurred since the first GC/MS instrument was built. Frequently, a computer can inject a sample, take the data, processes the data, and print out a report without any operator intervention.

Since the introduction of capillary columns in 1980[32], most GC/MS instruments were converted to their use. These columns are 10 meters or greater in length, 0.23 to 0.32 mm in diameter, and are prepared from fused silica to provide inertness. The inertness of fused silica capillary columns frequently allows separations to occur without derivatization whereas packed columns would require derivatives of polar or basic molecules to provide the proper peak shape.

The GC inlet is a few PSI over atmospheric pressure whereas the mass spectrometer operates in the 10^{-6} Torr range. Since the flow rate though a capillary GC column is low, the GC column is led directly into the mass spectrometer without an interface. Earlier packed columns and wide bore capillary columns require a splitting valve or separator to either divert or remove some of the carrier gas. Direct connection allows maximum sensitivity since no sample is lost in the interface. Since everything injected into the GC elutes into the mass spectrometer, materials with low-volatility can contaminate the mass spectrometer. This necessitates frequent cleaning.

The mass spectrometer consists of three main parts: (1) an ion source to ionize the molecules entering the system, (2) an analyzer to separate the ions and determine their mass/charge ratio, and (3) a vacuum system which houses both the ion source and the analyzer since ions cannot be conveniently handled at high pressures.

In the ion source, ions are created from the molecules by bombarding them with 70 eV electrons. This imparts enough energy to the molecule to both ionize and fragment the molecule. This forms characteristic molecular and daughter ions. Alternative ionization techniques, such as chemical ionization, create ions by charge exchange. These are not frequently employed for drug analyses since fragments are not produced. The abundances and mass/charge ratios of the daughter ions aid in identifying the drug.

Representative mass spectra of codeine and morphine are shown in Figure 6. Even though their structures only differ by the methyl group on the phenolic oxygen, their mass spectra are significantly different. This characteristic fragmentation pattern makes mass spectrometry definitive.

Figure 7a shows a total ion chromatogram (TIC) of a mixture of codeine and morphine. A TIC is generated by the computer which plots the sum of all ion abundances in each scan versus time or scan number. The TIC is a reflection of the elution of materials from the gas chromatograph. Each scan contains the complete mass spectrum of the compounds eluting from the GC at that point in time.

A complete mass spectrum is normally not taken. Only selected ions which represent the compounds identified by the initial screen are used. Three representative ions of codeine are shown in Figure 7b. As can be seen in Figure 7b, only the codeine molecule has all three ions. The mass spectrum of morphine only contains the ion at mass/charge 162.

To confirm a compound by selected ion monitoring the criteria of Table 5 must be met.

Table 5 - Criteria for Positives by GC/MS

- Proper retention time
- Gaussian peak shape
- Proper ion ratios
- Quantitation above cut-off

Selected ion monitoring increases the sensitivity of the mass spectrometer since more time is spent examining the ions of interest. Examining only a few ions also lowers the background since some compounds are ignored as in the example of morphine in the selected ion traces in Figure 7b.

To analyze a compound by GC/MS it must be volatile enough to elute from a GC column without decomposing. Since most drugs of abuse are small molecules, they have enough volatility to be analyzed with preparing derivatives. However, sometimes derivatives are prepared to improve the chromatography or provide a better separation from interfering species. For some low mass compounds such as amphetamines, derivatives are used to provide higher mass ions that are more characteristic and have fewer interferences.

A serious limitation on mass spectrometry is that it cannot distinguish between optical isomers. Optical isomers have identical physical properties including their mass spectra. Therefore, the optical isomers of methamphetamine, as mentioned in the section on cut-off levels, would be indistinguishable by mass spectrometry. Also, since few uses are made of columns that can resolve optical isomers[33,34], the retention time of these two optical isomers would be identical.

Generally all optical isomers of drugs of abuse are considered to have the same activity and illegality by the Federal Code of Regulations. Cocaine is an exception. Only the natural optical isomer, l-cocaine, is illegal to possess. Legal defenses based on incomplete identification of the optical isomer are usually not successful.

PITFALLS IN TECHNOLOGY

There are two major problem areas for mass drug screening. One is the collection of the sample where adulteration could take place. Observing the person providing the sample and disciplining the observer if adulteration is found can minimize this problem. Careful attention to chain of custody must occur to avoid mix-up of a sample.

Once the sample reaches the testing laboratory, precautions must be taken to insure testing of the proper sample. Since instruments can become contaminated by high-drug levels from a previous sample, carry over to the next sample is a possibility. This has been observed in the Navy screening program. The Navy screens all positive samples twice by RIA before testing by GC/MS. In the second RIA screen there are blank bottles between samples to minimize carry over. Also any positive samples are retained. Retention of samples will be required by civilian testing guidelines[4]. If an individual suspects an error may have occurred, he/she may request a retest of his sample either by the Navy laboratory or a private laboratory.

(Enzyme Multiple Immunoassay technique)

SUMMARY

(Cont'd) (Thin Layer Chromatography) (Radioimmunoassay) (Fluorescent Polarization Immunoassay)

Four mass screening techniques for drugs of abuse (TLC, RIA, EMIT and FPI) have been described. For small-scale screening, TLC is the most cost effective. It cannot achieve the sensitivity of the other three immunologically-based techniques, but it is sufficient for most deterrent purposes. All the screening techniques are subject to interferences that can generate false negatives. Of the four described, EMIT is the most susceptible to interferences.

If the quantity of a drug is above a cut-off level by the screening test, then the presence of the drug is confirmed by GC/MS. Selected ion monitoring is used in the GC/MS confirmation to increase the sensitivity and decrease interferences. In GC/MS, the quantity of the substance also must be above a cut-off level for a sample to be positive. If all criteria of drug presence are met by two independent techniques, then that sample is confirmed to contain a drug of abuse.

Any testing program must undergo constant supervision to insure accurate results. Any errors found must be corrected and the protocols modified so that those errors are eliminated in the future.

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32. Fused silica capillary columns were patented by Hewlett-Packard Co. in 1980. c.f. Chem Abs. 94:76257a.

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35. Toxi-Lab is a trademark of Analytical Systems, Divison of Marion Laboratories, Inc., Laguna Hills, CA 92653. EMIT is a tradmark of Syva Corporation, Palo Alto, CA 94304. TD_x is a trademark of Abbott Laboratories, Irving, TX 75015. Vicks Inhaler is a trademark of Vicks, Inc., Wilton, CT 06897.

Figure 1 - Principle behind radioimmunoassay.

Figure 2 - (a) Typical radioimmunoassay results plotted as raw data. (b) Raw data plotted with a function designed to generate a straight line.

Figure 3 - Enzyme reaction used in enzyme multiplied immunological technique.

Figure 4 - Principle behind EMIT.

Figure 5 - Principle behind fluorescent polarization.

Figure 6 - Mass spectra of codeine and morphine.

Figure 7 - (a) Selected ion traces for a mixture of codeine and morphine. (b) Total ion chromatogram of a mixture of codeine and morphine.

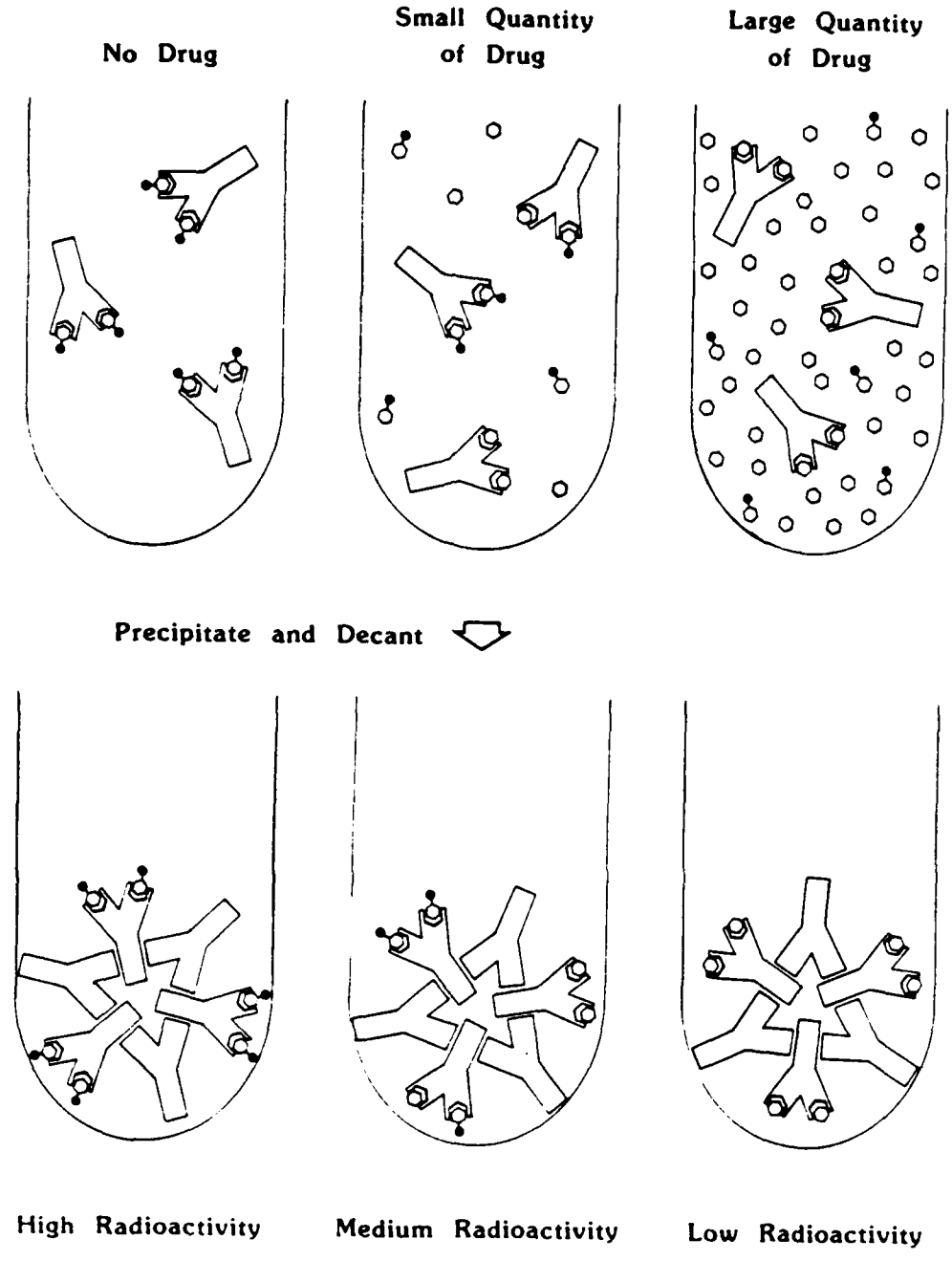
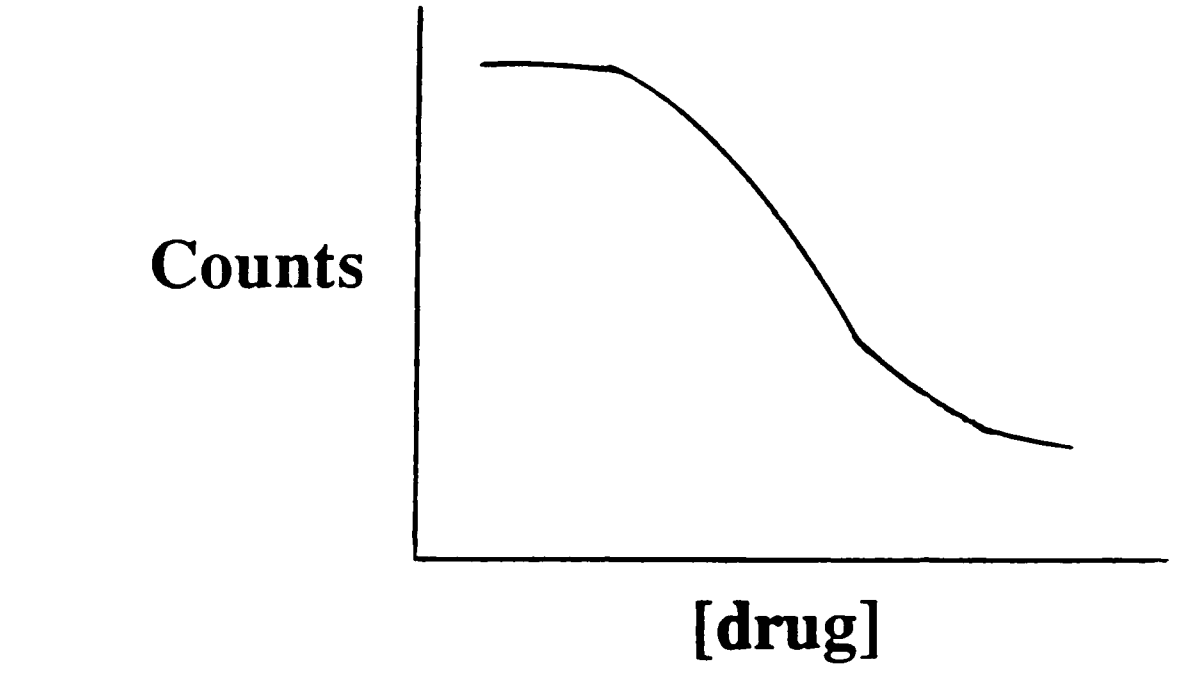

No Drug
Small Quantity of Drug
Large Quantity of Drug
Precipitate and Decant
High Radioactivity
Medium Radioactivity
Low Radioactivity
Key:
First Antibody
Second Antibody
Drug
Radioactively-Labeled Drug

FIGURE 1



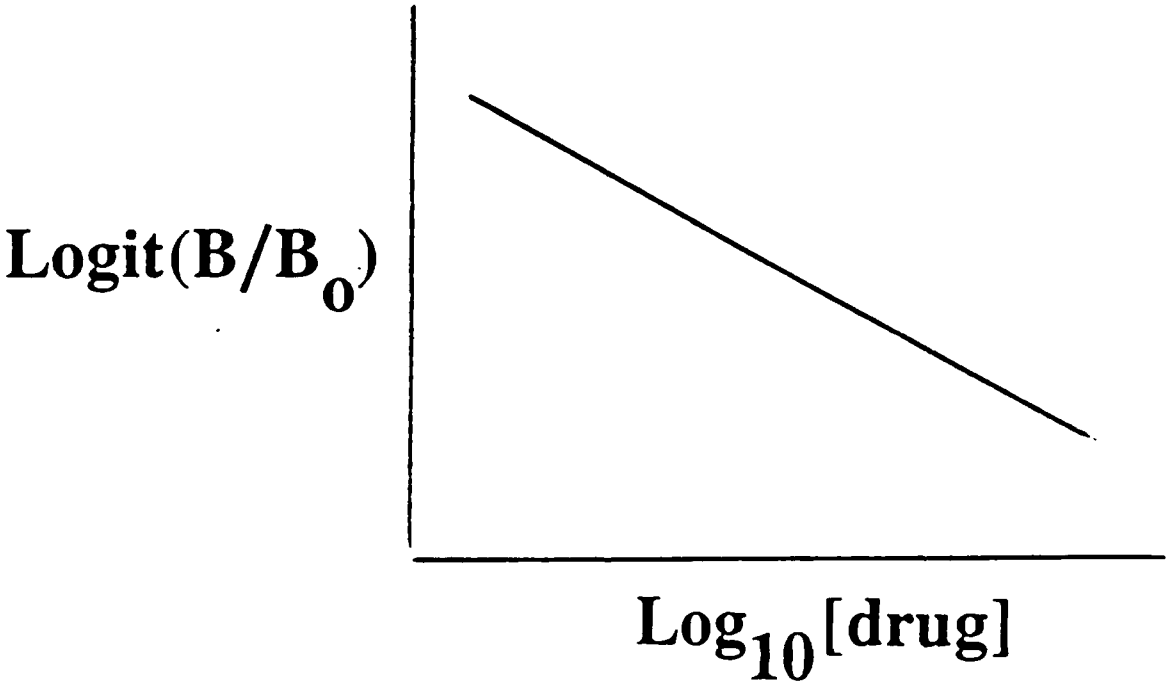
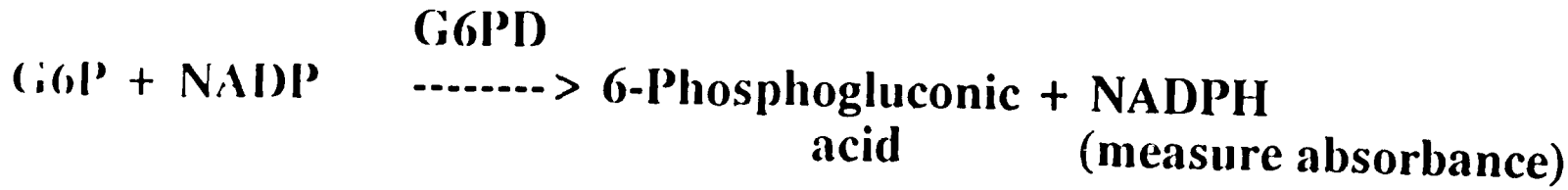
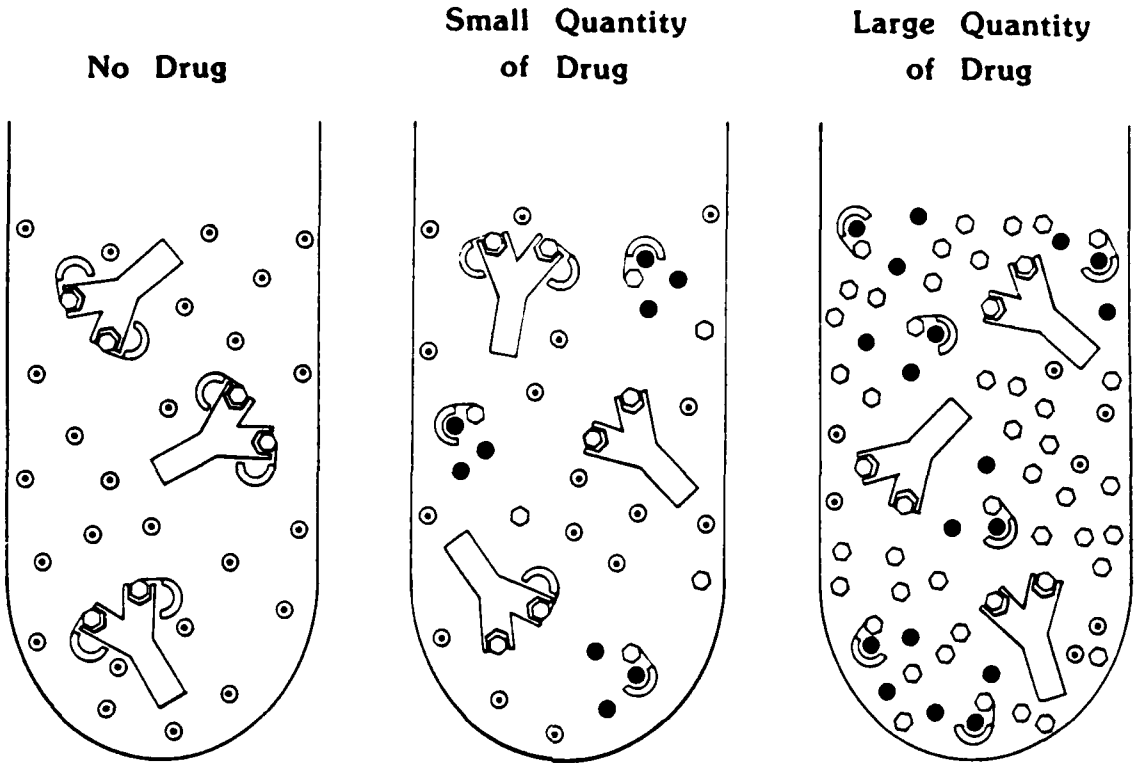


FIGURE 2



G6P = Glucose-6-phosphate
G6PD = Glucose-6-phosphate dehydrogenase
NADP = Nicotinamide adenine dinucleotide phosphate
NADPH = Reduced Nicotinamide adenine dinucleotide phosphate

FIGURE 3


No Drug
Small Quantity of Drug
Large Quantity of Drug

Key:


Antibody
Drug
Enzyme-Labeled Drug
Enzyme Substrate
Degraded Substrate

FIGURE 4

Fixed rotation
Short lifetime
Free rotation
Long lifetime
Complex has
hindered rotation
Drug
Antibody
Complex

FIGURE 5

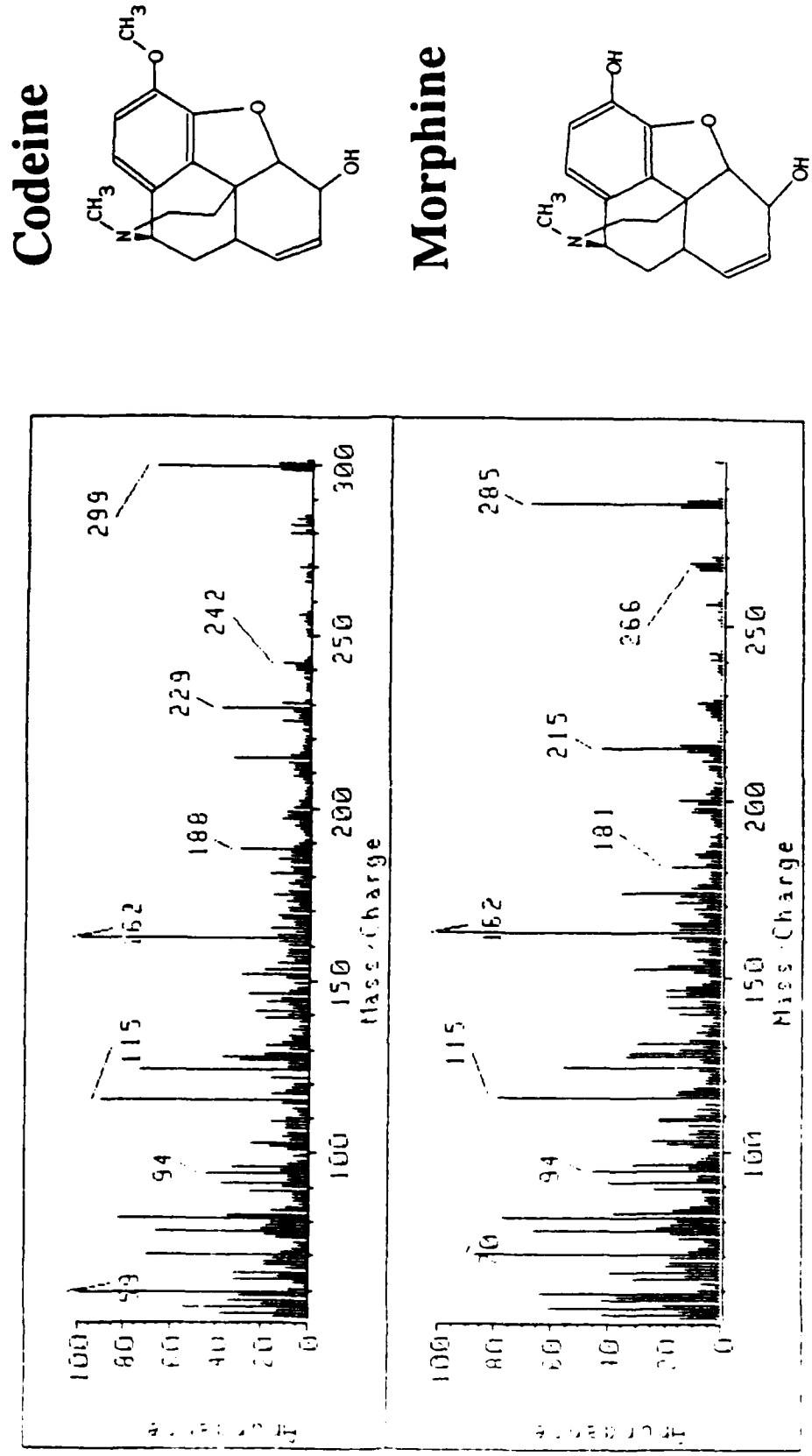
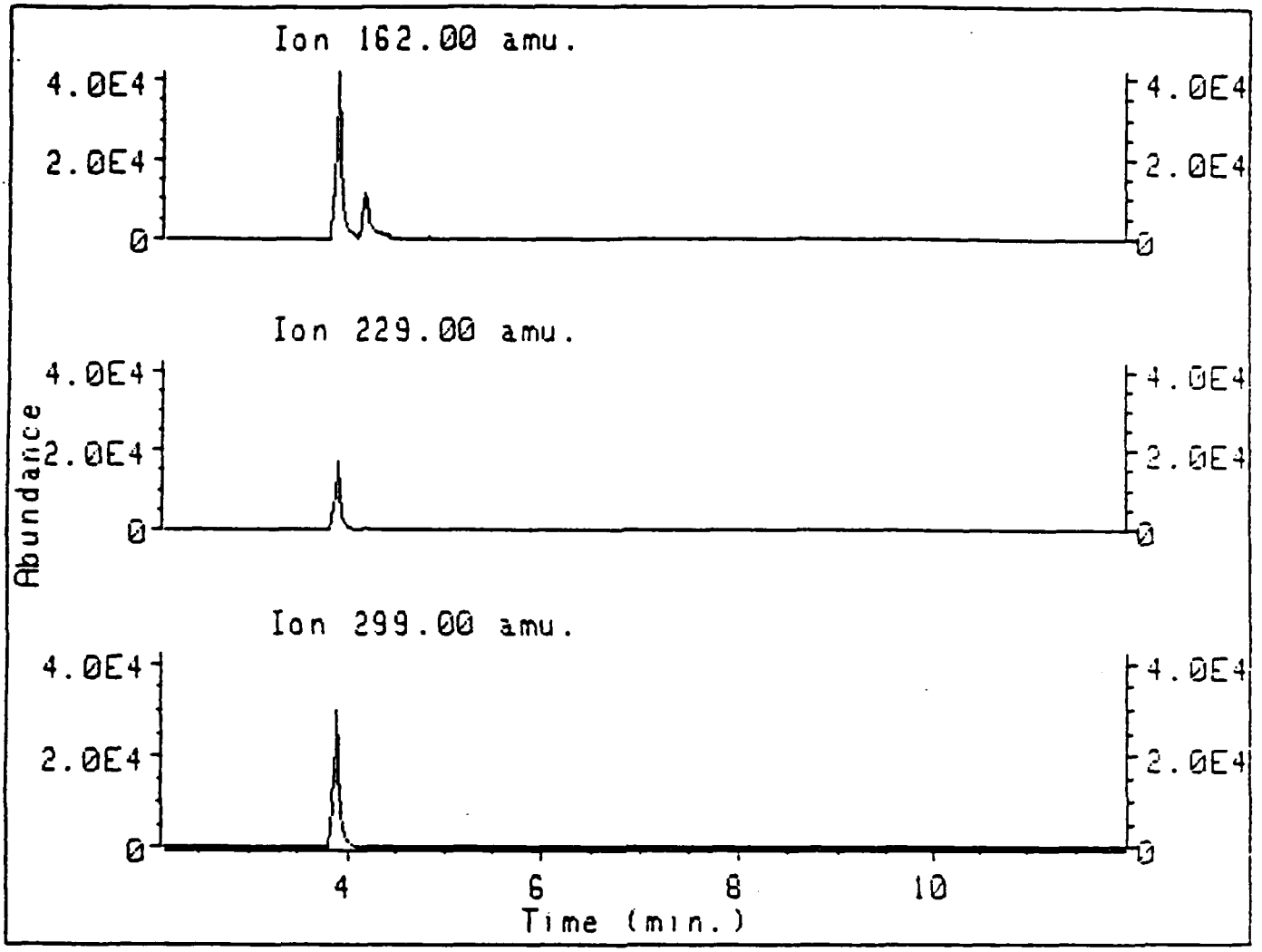

Codeine
Morphine
299
242
229
188
162
115
94
285
266
215
181
Mass/Charge

FIGURE 6


Ion 162.00 amu.
4.0E4
2.0E4
0
Ion 229.00 amu.
Abundance
Ion 299.00 amu.
4
6
8
10
Time (min.)

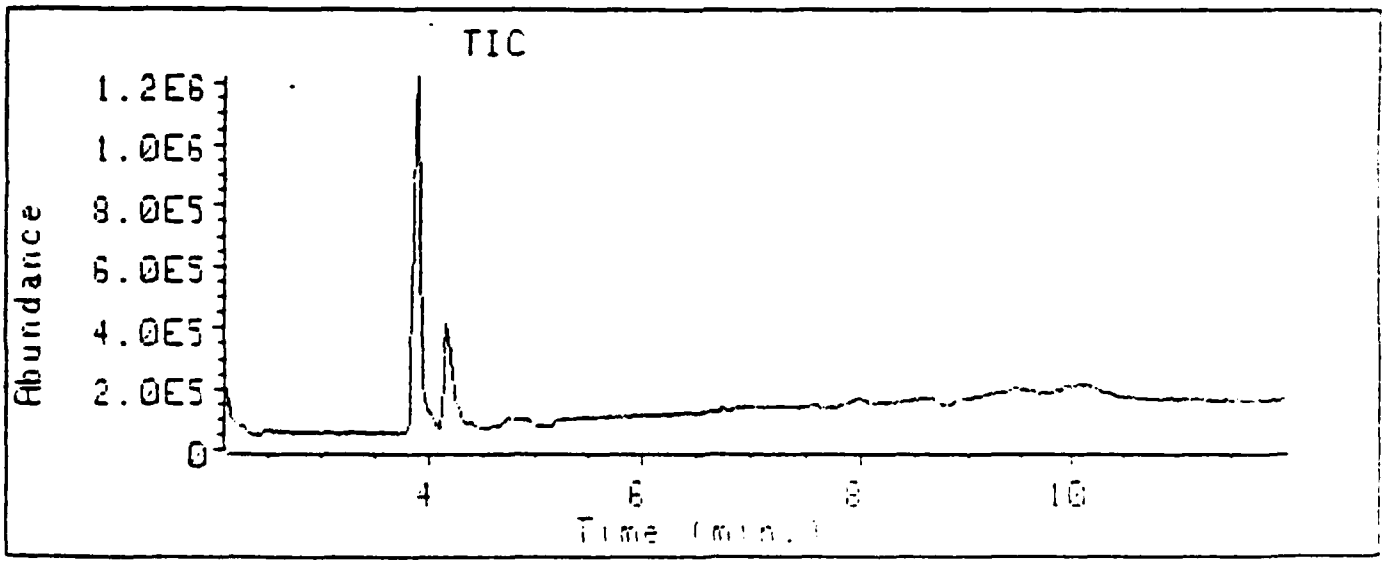

TIC
1.2E6
1.0E6
8.0E5
6.0E5
4.0E5
2.0E5
0
Abundance
4
6
8
10

FIGURE 7

END

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